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EFFECTS OF IMPOUNDMENTS ON WATER QUALITY IN THE SOUTHWEST. DISCUSSION - BACTERIAL CYCLES AND WATER QUALITY

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Discussion —

BACTERIAL CYCLES AND WATER QUALITY

by DR. RUFUS K. GUTHRIE*
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North Texas State University
Denton, Texas

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IN the determination of bacterial counts for the detection of cycles of presence in a large body of water, one must take into account several sources of error. One occasional happening which may produce major dislocations of bacterial presence is a period of heavy rainfall, producing extensive runoff into the water impoundment. Such drainage increases the total bacterial count for a period of one to two weeks and must be taken into account when looking for bacterial cycles which may occur in the water.

After studying bacterial presence in one water impoundment for a period of 1½ years, we have come to the conclusion that this influx of bacterial population does not alter the overall pattern of cycles which may be present. Total counts are increased by the runoff, and relative numbers of different groups are increased in approximately equal proportions. Following all counts drop off proportionately.

The occurrence of cycles of bacterial presence can be detected if one accounts for such periodic disturbances. In Figure 1 we show the results of consecutive periodic counts of bacteria over a period of approximately 15 months. In this figure we can see the higher counts occurring at times when

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MARK XIII

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*Read before Southwest Section, AWWA Convention held at Oklahoma City, Okla., Oct. 1967.

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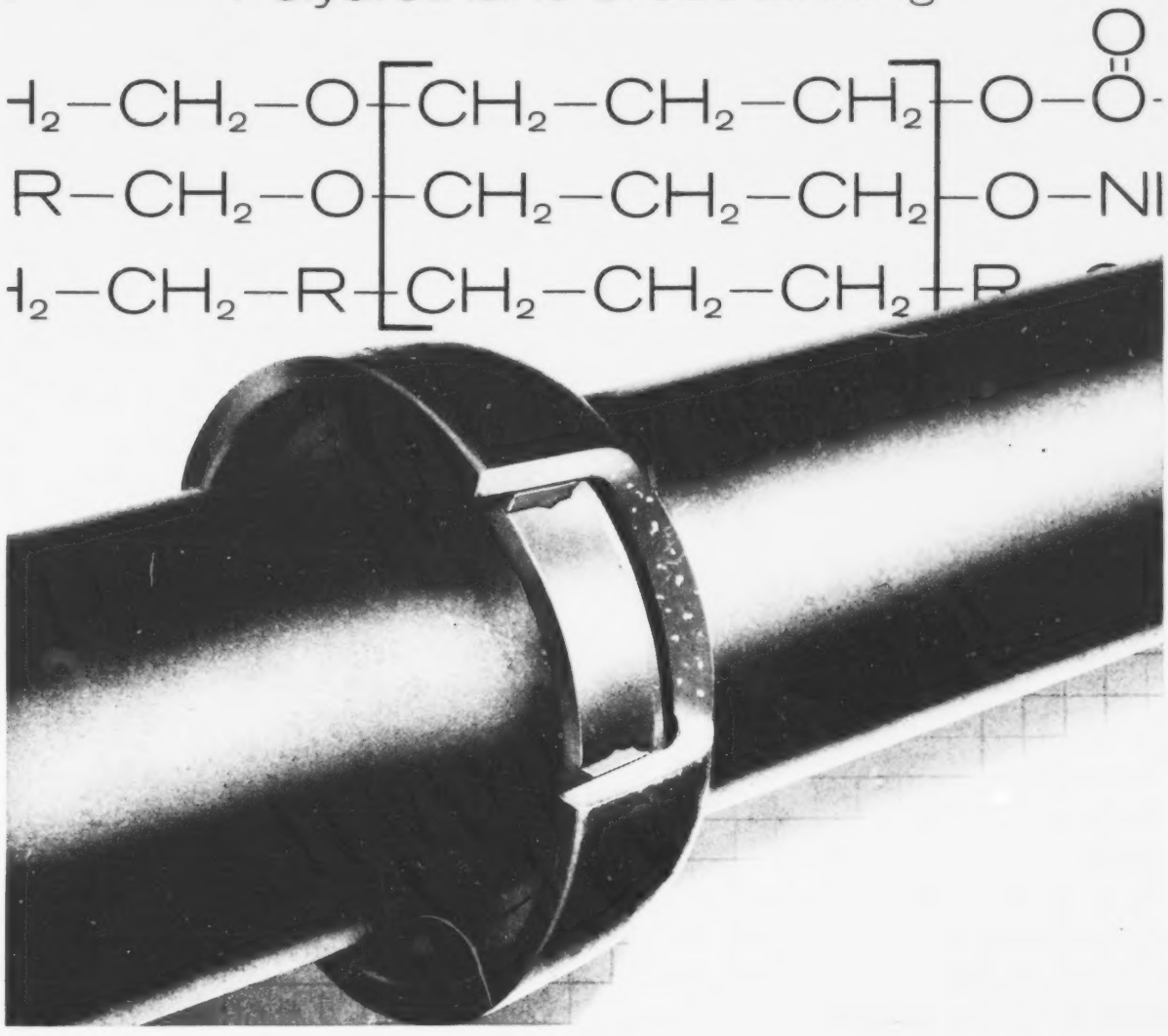

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we expect heavier rainfall—June '66, December '66, February '67, June '67. To detect cyclic presence then we must consider relative proportions in group populations, shown in Table 1.

In consideration of these data we have arbitrarily designated any presence above 5% as being a major segment of the total population. If this is done and we look for seasonal patterns, we can designate the cool months as a time of high presence of gram negative organisms. (These include the *coliaerogenes* groups, *Alcaligenes*, colon-like organisms, *Achromobacterium*, and soil forms such as *Azotobacter* and *Rhizobium*.) The warmer months show a predominance of gram positive organisms—(*Bacillus, Brevibacterium, micrococci, Sarcina, Gaffkya,* and *Corynebacterium*).

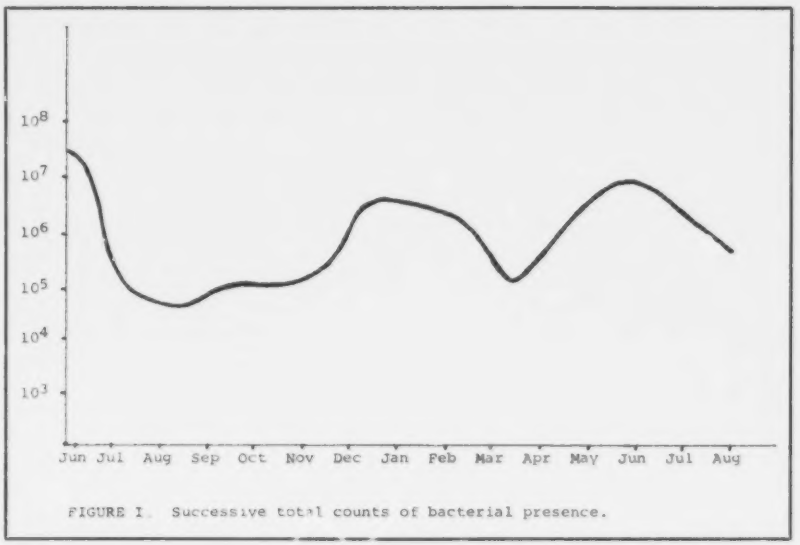

FIGURE I. Successive total counts of bacterial presence.

Groups which show no pattern of presence are gram negatives—*Flavobacterium* and *Pseudomonas*, and gram positives—*Brevibacterium*. The fungal presence is one which must be considered with some skepticism, because of the nature of the organisms, and particularly because of the ease of spread and contamination with fungal spores. Apparently, the fungal presence parallels closely the presence of bacilli. Whether this is meaningful or not, we cannot say at this time.

In these results we cannot assign great significance to the continued high presence of *Bacillus* species, since these organisms were added to the lake in the warm months. We can say that these bacteria were able to maintain a high presence in association with the other micro populations of the water; however, a permanent predominance was not established. One feature of these data is noteworthy, in that an extremely high presence of *Bacillus* is in most cases accompanied by a high presence of other gram positives, and a drop in gram negatives of all types. The reverse is true of a high presence of gram negatives of any variety. A high presence of *Flavobacterium* is always accompanied by a high presence of other gram negatives with these periods then being followed by shifts to high presence of bacilli and other gram positives.

These cylic shifts do not appear, nor indeed to the relative proportions of bacterial presence appear to be greatly affected by the influx of water in high runoff periods which can be detected by the highest total counts. We do detect an increase in the presence of fungi following an influx of runoff.

We have not yet mentioned the Actinomycetes. These organisms show no particular pattern of presence. We do note that the presence was higher in July and August 1966 than at any other date, and the summer of 1967 did not show a repeat of this increased incidence in that the presence of Actinomycetes never reached a 5% level after February 1967. Yet relative presence figures of other bacteria of summer 1967 again approximated the figures of 1966.

With these data we may speculate on the chemical events which affect water quality. I think we must begin with the prediction that this water is of consistently good bacteriological quality. This conclusion is not based on the total absence of any proven pathogens or the absence of *E. coli*. Rather we would base this statement on the fact that we do have at all times a good mixture of bacterial populations, and that all members of this mixture are able to maintain continual presence. We have observed no loss of population segments for appreciable lengths of time, and we have observed no predominance of any population segment. The addition of one segment (*Baccilus*) did not allow the elimina-

Table 1. Percentage of bacterial groups in successive samples.

	1966									1967				
Month #	↑6	7	8	9	10	11	↑12	1	↑2	3	4	↑6	7	8
Bacillus	22	14	20					8			7	11	40	10
Fungi	34	10	40					8		7		6	20	10
Actinomyces		5	9	10					5					
Pseudomonas						11					5	10		
Flavobacterium	5	20	6		6		26			27	19	19		5
Brevibacterium	5	14				30		16				6		10
Gram - Rods	15	25	16	76	46	37	65	51		51	42	34		41
Micrococci	5	5	6	5	41		27						15	10
Other Gram +	5	6	5	8			5			5	22	12	20	10

↑Periods of highest total bacterial counts.

"You'll catch on, but I think it would be safer if you'd forget about all you've learned about riding a motorcycle!"

tion of any other group. A cyclic balance is observed in this particular ecosystem.

In the water as pollutants, we may expect to find included the following assortment of organic chemicals: carbohydrates, hydrocarbons, and various assortments of proteins or protein source compounds. *Bacillus, Aerobacter, Micrococcus, Sarcina, Gaffkya,* and various strains of *Escherichia* are capable of fermentation of a wide variety of carbohydrates. This fermentation will be accompanied by the production of an assortment of chemical by-products including some of the essential growth factors, which will serve as nutrients for other microflora.

Hydrocarbons, not expected in large quantities here will be metabolized by a variety of bacteria, including *Pseudomonas* species and various soil forms. Products of this metabolism will in turn be used by other microorganisms.

Gram negative bacilli, *Brevibacterium* and others will metabolize proteinaceous materials and thus will assist in breakdown of cell bodies. Many soil forms also assist in this process, sometimes with the production of ammonia, hydrogen sulfide, and putrefactive odors. However, with a balanced micropopulation, the metabolism here should be relatively complete and these obnoxious products will thus be avoided.

With this assortment of metabolic activities and the maintenance of cyclic bacterial population presence, the water quality is maintained at a level which makes it desirable for human uses. This quality involves both chemical and bacterial factors. As long as the quality remains good, we can expect the maintenance of bacteriological cycles, which maintain the chemical quality. If we overload the chemical quality in any direction we will permit the predominance of certain bacterial groups to the exclusion of others, and eventually in this manner, will further increase the imbalance and produce water unfit for human uses.

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WPD-146

Demonstration Grant
WPD-146

from

United States
Department of the Interior
Federal Water Pollution Control Administration

for

Period

December 1, 1966 - August 31, 1969

Prepared by

Rufus K. Guthrie, Ph.D.
Professor of Biology
North Texas State University
Denton, Texas

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Summary Statement

The first two years of this grant period was used to collect data on relative presence of microbial populations in a fresh water impoundment and to develop reagents and refine serological techniques for use in a membrane filter-fluorescent antibody method of bacterial identification and enumeration. The first of the steps above is summarized in the publication "Bacterial Cycles and Water Quality"; SWWJ, 50:54-56, 1968. Twenty-five reprints of that work are included with this report. The data is being prepared in more complete form for submission for publication, but requires revision before acceptance. This preliminary, more complete preparation is attached as Appendix A.

The second of the steps above involved production of stocks of specific antiserum and conjugation of this antiserum with fluorescent dyes for use in the membrane filter-fluorescent antibody tests. Reagents in this period were prepared and preliminary specificity testing was completed for nine species or strains of Streptomyces and for twelve strains of Escherichia coli.

Results

The detailed results from the first phase of the work above are given in the enclosed reprints and in Appendix A. These will not be repeated here.

Because of the problems generally associated with them, two

groups of bacteria were chosen as organisms for initial studies: the Actinomycetes and coliforms. The Actinomycetes, primarily Streptomyces, have been repeatedly shown to produce unpleasant tastes and odors in water supplies by the work of Dr. J. K. G. Silvey and others. Escherichia coli, of course, is the indicator organism for pollution. The possibility of early and rapid identification and enumeration of these organisms is of obvious desirability.

To study the possibility of using a fluorescent antibody-membrane filter technique for identification and enumeration of Streptomyces species in water, antisera were prepared for 3 strains isolated locally, and for 6 species or strains obtained from stock culture collections. Multiple injections of the cell walls from broth cultures of Streptomyces served to stimulate antibody production to a practical level in rabbits which were bled periodically and the serum harvested and pooled. Pooling of the serum was found to be satisfactory for the production of broad specificity antiserum.

The pooled serum was treated by saturated ammonium sulfate to precipitate and concentrate the gamma globulin fraction. The dialyzed globulin concentrate was conjugated with fluorescein isothiocyanate by the method of Marshal, et al. (Proc. Soc. Exp. Biol. and Med., 1958. 108:898-900). Following conjugation, the

serum was passed through a Sephadex G-25 column to remove the excess dye, and then stored at 5°C. for use.

Initial tests were made using laboratory stock cultures of Streptomyces diluted 1:10,000 in tap water. This diluted suspension of cells was filtered through a sterile millipore filter of 0.45 micron pore size of type HABG. The HABG was chosen after test of five different filters from two manufacturers as being the filter of least autofluorescence which could be manipulated in this method without damage. Following passage of a sample of the cell suspension through the filter, the filter was removed and placed immediately on the surface of a tomato juice-oatmeal agar plate. The plates were incubated for 18, 24, 48, and 81 hours to allow growth of colonies. It was found that incubation times shorter than the 18-hour period would not allow colony formation of a size to be visible at the magnification used in this work. Following incubation, membranes were flooded with the gamma globulin-fluorescein conjugate for a time sufficient to allow specific reaction. The excess conjugate was removed by washing with buffered saline, the membranes dried, and were then ready for observation. Normal serum, and specific antibody controls were run in parallel with each test. The membranes were scanned using a low power dissecting scope with a B & L mercury power supply and 200 watt mercury arc lamp and suitable filters.

A top-lighting technique at a 30° angle was used. All counts using this membrane technique were duplicated by standard plate counts.

By using these methods, representative results were obtained that are shown in Table 2. In repeat runs, using mixtures of local isolate Streptomyces as test organisms, additional data was obtained. Table 3 shows results of one such experiment.

In these tests, we have demonstrated the usefulness of this means of rapid detection of the presence of Streptomyces species in water samples. Improvement in the efficiency of the method is being sought by refinement of techniques. The breadth of specificity of the conjugated serum used appears to be fairly good; however, some improvement in this factor would perhaps be important in increasing the efficiency. These tests appear to reduce the time required for recognition and quantitation of Streptomyces colonies from 7-10 days to 48 hours, and in this respect, it is felt that the method is worthy of additional attention with the view of refining techniques.

The common use of E. coli as the bacterium made the study of this organism imperative for evaluation of this method. Of twelve strains for which antisera were prepared, eleven antisera proved specific and useful with this method. The processes used in preparation and testing of these antisera are described

TABLE 1. Quantitative Determination of *Streptomyces* from Dilution Samples.

Colony Location	Dilution	Direct Colony Count*	# of Cells/ml by Direct Colony Count**	Ultra-violet Colony Count*	# of Cells/ml by U.V. Colony Count**
Membrane Filter	1:1000	12	1.2×10^4	13	1.3×10^4
	1:2000	5	1.0×10^4	5	1.0×10^4
	1:4000	5	2.0×10^4	6	2.4×10^4
	1:8000	1	8.0×10^3	4	3.2×10^4
Spread Plate	1:5000	2	1.0×10^4	--	----
	1:10,000	4	4.0×10^4	--	----
	1:20,000	.3	6.6×10^3	--	----
	1:40,000	.3	1.3×10^4	--	----

* These figures represent the average number of colonies per plate from three plates.

** These figures are to the nearest thousand.

TABLE 2. Quantitative Determination of *Streptomyces* Cells from Dilution Samples.

Colony Location	Dilution	Direct Colony Count*	# of Cells/ml by Direct Colony Count**	Ultra-violet Colony Count*	#of Cells/ml by U.V. Colony Count**
Membrane Filter	1:300	573	1.7×10^5	455	1.4×10^5
	1:600	25	1.5×10^4	56	3.4×10^4
	1:1200	18	2.2×10^4	46	5.5×10^4
	1:2400	10	2.4×10^4	14	3.4×10^4
Spread Plate	1:1500	193	2.9×10^5	--	----
	1:3000	20	6.0×10^4	--	----
	1:6000	15	9.0×10^4	--	----
	1:12,000	5	6.0×10^4	--	----

* These figures represent the average number of colonies per plate from three plates.

** These figures are to the nearest thousand.

in full in the 25 accompanying reprints "Membrane Filter-Fluorescent-Antibody Method for Detection and Enumeration of Bacteria in Water", Appl. Microbiol., 17:399-401, 1969. These results will not be repeated here, however, the conclusions, based on the above, which resulted from that work are: (1) The Membrane Filter-Fluorescent-Antibody (MFFA) Method described compares favorably with standard methods now in use as to accuracy of detection and enumeration of total coliforms and of fecal coliforms. (2) The MFFA method has one great advantage in shortening the time required for complete testing to 12 hours maximum, as compared to 18 to 72 hours for other standard methods.

Following the above work with E. coli and to some degree concurrent with it, additional studies were undertaken to expand tests for applicability of the MFFA method for determination of other bacterial types in water supplies, and to reduce the time required for identification and enumeration of E. coli below the 12 hours previously reported.

In the time studies using E. coli as the test organism, repeated tests have shown that the test can be reduced to 6 hours with considerable loss in accuracy and in specificity. A major problem encountered in these tests was flotation of colonies and a failure to stain in some of the very young cultures. As shown in Table 3, however, the time can be reduced to 10 hours with no loss of sensitivity or accuracy.

TABLE 3. The Reduction of Incubation Time Required for Enumeration of *Escherichia coli* by the MFFA Method.

	Dilution (Neph.)	Time hrs.	Count (x2)	F. A. Reaction
E. coli #9	10^8	12	500+, 500+	3+
	10^9	"	240, 270	"
	10^{10}	"	75, 79	"
	10^{11}	"	30,25	4+
E. coli #9	10^9	10	TNTC	1+
	10^{10}	"	90, 100	"
	10^{11}	"	40, 25	±
E. coli #9	10^{12}	10	12, 13	3+
E. coli + Strep	"	"	26, 35	4+
E. coli + Beer	"	"	18, 8	3+

The use of the MFFA method for other bacterial species has been concentrated in studies dealing with Bacillus cereus and with Streptococcus fecalis. The rationale for this concentration has been as follows: In separate studies at North Texas State University, it has been determined that the addition of B. cereus cultures to water supplies will reduce or eliminate certain undesirable tastes and odors. It is therefore desirable to have a method for rapid and accurate monitoring of the presence of this bacterium. S. fecalis was studied as a potential indicator organism for fecal pollution, particularly in waters to which chlorinated sewage is added.

The detailed results of work with Bacillus cereus and related species is attached in Appendix B.

Detailed results of work with Streptococcus fecalis is attached in Appendix C.

The project director, Rufus K. Guthrie, Ph.D., spent full time on this project for six weeks in the summer of 1967 and the summer of 1968. He has spent approximately 15% of the remainder of his time on this project to August 31, 1969. As of September 1, 1969, the project director will change his location to Clemson University and will become Director of the Division of Biology at that institution in Clemson, South Carolina. Since Dr. J. K. G. Silvey has devoted considerable effort to consultation and assistance

with this project and is thoroughly familiar with the work and the rationale behind it, it is respectfully requested that this project be continued under his direction at North Texas State University until its completion and termination. He will have available, two graduate students who have worked for some two years on the project and who are continuing studies dealing with the MFFA method as used for detection of S. fecalis, and with a reduction of time required for completion of the MFFA tests. This continuation should do much to add to the information supplied under this grant project.

Reports and Technical Articles

1. "Identification of Streptomyces Species Using Fluorescent Antibody-Membrane Filter Techniques." J. David Singleton. Masters Thesis. 1967. Unpublished.

2. "Bacterial Cycles in Lake Hefner." John C. Stiles. Paper reported to local group at Oklahoma City. 1967. Unpublished.

3. "Bacterial Cycles and Water Quality." Rufus K. Guthrie. Southwest Water Works Journal. 50:54-56. 1968.

4. "Fluorescent Antibody-membrane Filter Method for Specific Enumeration of Bacteria in Water." D. J. Reeder, and Rufus K. Guthrie. Reported to North Texas Biological Society, Fort Worth, (4-27-68) and to American Society for Microbiology, Detroit, Michigan, May 5-10, 1968. Abstracted in Bacteriological Proceedings, 68:34. 1968.

5. "Seasonal distribution of bacteria in a fresh water impoundment." R. K. Guthrie, J. C. Stiles, J. K. G. Silvey and K. Dickson. In Revision Preparation.

6. "A membrane filter-fluorescent antibody method for detection and enumeration of bacteria in water." R. K. Guthrie and D. J. Reeder. Applied Microbiology. 17:399-401, 1969.

7. "The Use of a Membrane Filter-Fluorescent Antibody Method for Specific monitoring of Bacillus cereus in Water Supplies. Robert Ferebee. Masters Thesis. 1969. Unpublished.

Appendix A.

Seasonal Distribution of Bacteria in a Fresh Water Impoundment

R. K. Guthrie, J. C. Stiles, J. K. G. Silvey, K. Dickson
Biology Department, North Texas State University, Denton

ABSTRACT: The aerobic bacterial populations of a fresh water reservoir were monitored for a period of two years to determine fluctuations in total counts as well as the relative percentage of selected bacterial species and groups. Standard plate counts and culture procedures were used to enumerate and identify bacterial cultures. Populations of bacterial groups exhibited seasonal variations which were repeated in the period of study. Periods of high predominance by one group of organisms were never maintained for successive test periods. Factors influencing bacterial population levels are discussed.

Seasonal Distribution of Bacteria in a Fresh Water Impoundment*

R. K. Guthrie, J. C. Stiles, J. K. G. Silvey, K. Dickson
Biology Department, North Texas State University, Denton

The bacteriology of lakes and reservoirs has been of interest primarily from the standpoint of potability, and occasionally in reference to specific groups, genera or species of bacteria as concerned with specialized activities. The relative number of genera of bacteria present in natural water sources has been of little general interest in the past, and consequently, little is known of the distribution, natural fluctuations or consistency of presence of bacterial populations in fresh water.

A number of reports have been made listing genera of bacteria present in various geographical locations in fresh water impoundments or lakes (Bloodgood, et al, 1959; Collins, 1963; Dratchev, et al, 1957; Fischer, 1959; Henrici, 1939; Kuznetsova, 1961; Malaney, et al, 1962). In these reports there are some genera common to most of the water sources studied. These include Pseudomonas, Azotobacter, Achromobacter, Flavobacterium,

* Supported in part by Grant WPD-146 from the Federal Water Pollution Control Administration, U. S. Department of the Interior, and in part by The Faculty Research Fund of North Texas State University.

Alcaligenes, Sarcina, Micrococcus and Brevibacterium.

In relation to a study of the use of chemicals for reduction of evaporation from water reservoirs, it became desirable to know the relative concentrations of aerobic bacteria present in a selected reservoir. To determine these concentrations and the stability of the bacterial populations, the following study was done.

Materials and Methods

All water samples tested in this work were taken from Lake Hefner, an artificial water impoundment located in Oklahoma City, Oklahoma. Sampling stations were established at five locations, from which test samples of 300 ml were taken at intervals of one week during summer months, and of one month during the cooler periods of the year. Sampling was continued for two years.

Within a period of two to four hours after sampling, the water was diluted appropriately and plated in 0.25 ml amounts on the surface of Standard Plate Count agar (Difco) plates. The 0.25 ml sample was spread uniformly over the plate surface with a flamed glass rod. Dilutions were predetermined to give plate counts between thirty and three hundred colonies. All samples were tested in triplicate at each of two dilutions.

Plates were incubated for five days at room temperature, (approximately 25°C.) to allow for growth of the maximum number of different aerobic bacterial types. Following incubation, total counts were made as differential counts based on colonial morphology and pigmentation. Colonies of each type were picked to new media for isolation to pure culture and for subsequent identification to the genus level. Media and tests used for identification were selected according to Bergey's Manual of Determinative Bacteriology, Seventh Edition. No attempt was made to detect or enumerate anaerobic organisms.

Although errors in initial sampling were obviously present in differential counts, with practice, accuracy in distinguishing separate types of colonies improved. As in any project dealing with mixtures of bacteria which must produce colonies before identification can be attempted, the relative numbers identified must be emphasized rather than absolute numbers.

Additionally, in a study of this type, the distortions present at a station at any one sampling time are likely to be excessive. One means of minimizing such artificial variation is the use of five sampling stations, and frequent repetition of sampling over a period long enough to permit detection of shifts in populations due to seasonal, weather, or other environmental changes.

Distortions may be expected following periods of heavy rainfall and runoff into a water impoundment. Resulting variations could be the result of addition of bacteria to give higher counts, or in dilution with consequent lower counts. Lake Hefner was chosen as the test reservoir because it is a man-made impoundment having a minimum of natural drainage, and the level of water is controlled at the single inlet which permits filling of the lake. (Bloodgood, et al, 1959; Henrici, 1939).

Results and Discussion

In preliminary sampling, bacterial populations were studied with the purpose of determining those genera or groups consistently present at all sampling stations. In these tests Bacillus, Pseudomonas, Flavobacterium, Brevibacterium, Micrococcus, and Streptomyces species were present in essentially all samples at all stations. In addition Alcaligenes, Achromobacterium, Azotobacter, Rhizobium, Aerobacter, Escherichia, Cellulomonas, Sarcina, Corynebacterium, Streptococcus, and Gaffkya species were found in occasional samples at one or two stations. Fungi and yeast forms were found in varying numbers, usually at one or two sampling stations at each sample time. From these tests grouping of genera was determined for presentation of differential count results.

The genera Bacillus, Pseudomonas, Flavobacterium, Brevibacterium, Micrococcus, and Streptomyces were identified and considered separately. The genera Alcaligenes, Aerobacter, Escherichia, Cellulomonas, Achromobacterium, Azotobacter, and Rhizobium were grouped as "other gram negative rods." Sarcina, Corynebacterium, Gaffkya, and Streptococcus species were grouped as "others." Fungi and yeasts were considered as one group.

Total bacterial counts are shown in Figure 1, varying from approximately 10^4 to 10^7 organisms per ml water. Such fluctuations in total bacterial counts can not be accounted for by rainfall or intake of water through the filling canal in every instance. The intake of water via canal, and monthly rainfall are shown in Figure 1, as acre feet of water. If these figures are compared to the total capacity of the reservoir, 2500 acre feet, it is possible to calculate the percentage dilution for any one period. If one looks at the dates 7-66, 4, 5, and 6-67, 9-67, 2-68, and 4-68, these are the times at which there was the greatest increase in water amount with the greatest resulting dilution. Yet, 7-66 shows one of the lower total counts; 4, 5, and 6-67 are relatively high total counts; and at the remaining dates, the total counts are in the middle range. Errors in sampling and plating, while obviously present in all cases, were

found to be consistent and could not account for such wide fluctuations in bacterial populations. One must assume then that the total bacterial counts are more affected by environmental factors other than those listed above.

The periods of highest total counts are seen in June, 1966, December, 1966, February, May, and August, 1967 (Figure 1). It is of interest to note in Table I that the relative percentages of groups making up these totals are not greatly different from relative percentages at other periods. This seems to indicate that those populations present in this reservoir are fairly stable, and that perhaps the best explanation for wide variations of counts would be weather conditions which accomplish mixing or stratification of the water.

Figures 2, 3, and 4 show the variations in group populations. In general those organisms shown in Figure 2 are at a peak population in the warmer months, decreasing in the cooler months to be replaced in predominance largely by gram negative organisms (Figure 3). The cycles of presence in these bacterial groups were repeated with good regularity at the same seasons in succeeding tests. Figure 4 indicates no repeated pattern of presence of these bacterial groups, with Micrococci and the heterogeneous "others" mixture being rather consistently present, while the

Brevibacterium group show wide variations ranging from absence to counts of 1×10^5.

The recurrence of cycles of presence over the two year test period (Table I. Figures 1-4), indicates the operation of a natural balance, not only among these organisms, but among all the lake biota. This reservoir is somewhat unusual in that there is little, if any, chance of pollution from human sources, with no natural drainage into the reservoir and prohibition of recreational use of the water. This permits the natural balance to be continued without interruption. The strength of the balance is indicated at certain periods when genera or groups reach a predominance at levels of 94% (Fungi, 8-66), 56% (Flavobacterium, 7-66), 70% (other gram negative rods, 7-66) and 76% (Bacillus, 10-67) and yet, within a few weeks these are returned to a more average concentration balance with other groups present. With this balance mechanism operating , it appears that the biological quality of the water should remain high.

Henrici (1939), in a review of bacteriological studies of natural lakes concludes that seasonal fluctuations of bacteria differ in different types of lakes dependent upon the regularity of turnover, drainage from the surface into the lake, pulsation and amount of plankton populations, and temperature. This review

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did not concern itself with seasonal fluctuations of specific groups or genera of bacteria, but rather with total populations of aerobic bacteria which could be cultured in the laboratory, or which could be stained and counted after adherance to glass slides submerged in the water. Those total counts reported are comparable to counts obtained in this study, ranging from 1×10^3 to 1×10^5 for culture studies and somewhat higher for direct microscopic counts.

The effects of biological and/or chemical pollution on the bacterial populations of a reservoir of this type must remain, at present, in the realm of speculation. Bacteria present have a great range of biochemical activities which conceivably handle a considerable amount of chemical pollution. Obviously, however, the biochemical capacity of these populations can be overwhelmed. If this should occur, only the hardiest, and not necessarily the most desirable bacteria will survive and will establish predominance. Consistent predominance of one group of bacteria would indicate poor water quality if we compare this segment of the environment to other situations in which only one living form has survived.

For water sources maintained at a quality level to be useful to man, this work appears to raise the question, "Can the biochemical

capacity of water biota be increased to a maintenence level, or once overwhelmed by pollution, can it be restored to a level sufficient to maintain natural biological cycles?" The answer awaits additional studies involving reservoirs and lakes of various pollution levels, with attempts at restoration of natural balance cycles by manipulating microbial populations.

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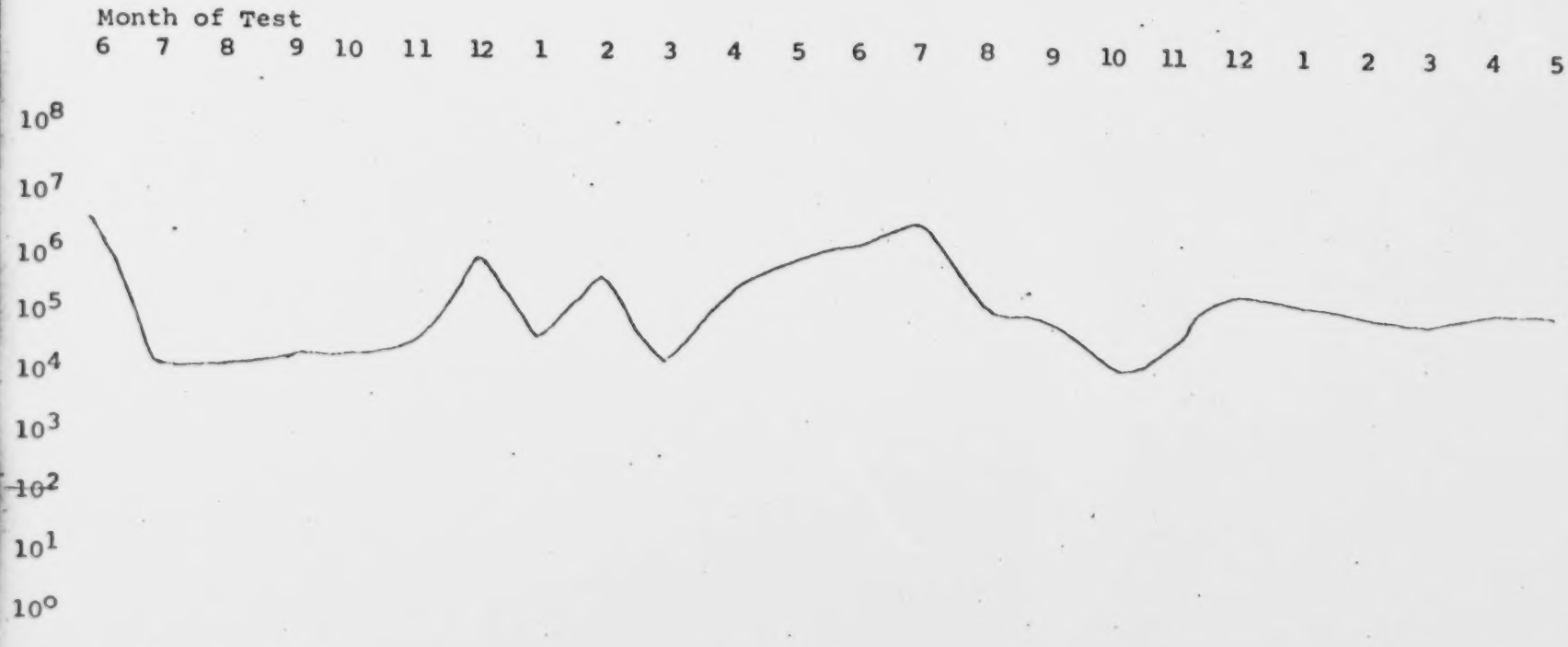
Total Counts, Figure 1



I	5	59	5	16	N	N	N	N	N	N	43	41	41	N	N	67	25	N	N	N	92	22	47	34
I%	.7	8	.7	1.5	N	N	N	N	N	N	6.1	6.0	6.0	N	N	9.1	3.5	N	N	N	12.2	30	64	5.0
R%	.8	.4	1.4	1.1	.14	.09	.19	.6	.06	.4	1.7	.9	.9	.5	.6	1.4	.8	.19	.3	.8	.4	.8	.8	1.8
A.F.	6.2	3.1	10	8	1	.6	2	4	.4	3	15	7	7	3	4	10	5	2	2	6	2	5	5	16

I = intake in hundreds acre feet
R = rainfall in acre feet

I% = percent dilution
R% = percent dilution

Figure II

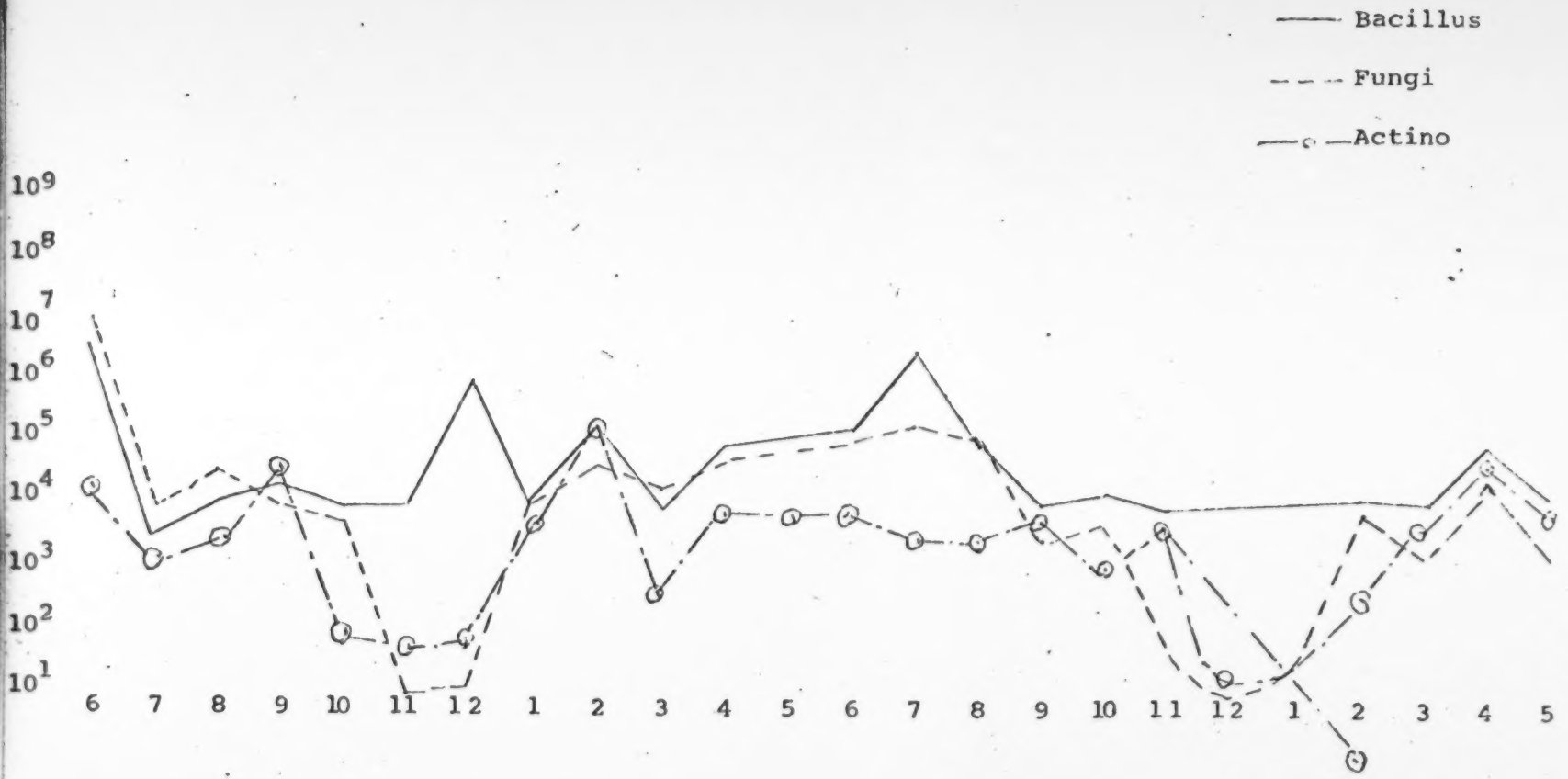

Bacillus
Fungi
Actino
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5

Figure III

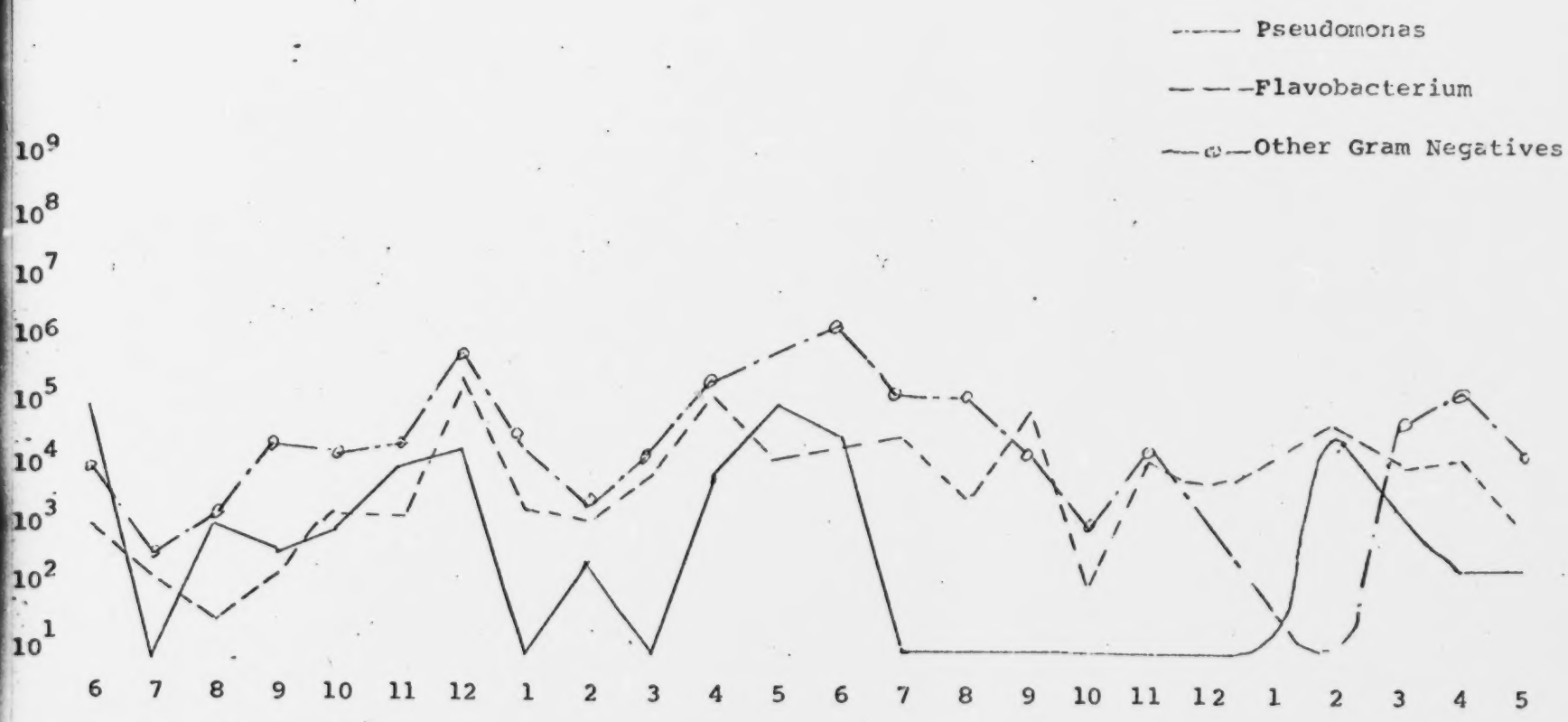


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Figure IV

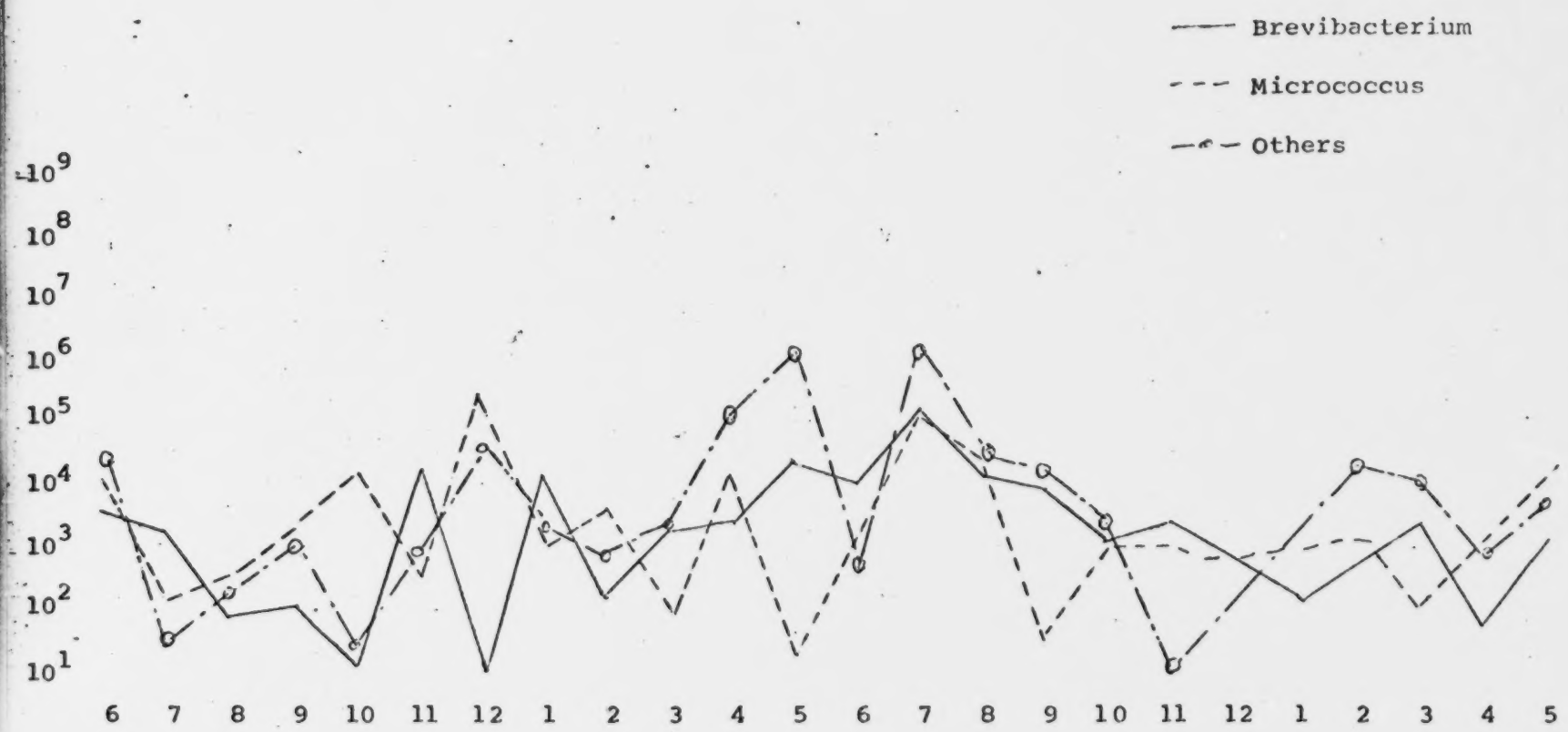
Brevibacterium
Micrococcus
Others
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5

Table 1. Percentage of bacterial groups in successive samples

	1966							1967											1968			
Month #	↑ 6	7	8	9	10	11	↑ 12	1	↑ 2	3	4	↑ 6	7	8	9	10	11	12	1	2	3	4	5
Bacillus	22	14	20					8			7	11	40	10	9	76	8			8			32
Fungi	34	10	40					8		7		6	20	10		6				6			5
Actino-myces		5	9	10					5						5		17						
Pseudo-monas						11					5	10								34			
Flavo-bacterium	5	20	6		6		26			27	19	19		5	48		18			33	10	13	
Brevi-bacterium	5	14				30		16				6		10	5		6						
Gram - Rods	15	25	16	76	46	37	65	51		51	42	34		41	21		47				71	79	30
Micrococci	5	5	6	5	41		27						15	10		6							15
Other Gram +	5	6	5	8			5			5	22	12	20	10	12	8				18	10		9

↑ Periods of highest total bacterial counts.

WPD-146

Appendix B

MATERIALS AND METHODS

Cultures for this work were obtained from the North Texas State University stock culture laboratory. These cultures are designated by numbers of the McBryde collection (MC), Dr. J. B. McBryde, Denton, Texas, the American Type Culture collection (ATCC), Washington, D. C., or the North Texas State University collection (NTSU), Denton, Texas. The following nine organisms were utilized in this work: Bacillus cereus, ATCC 10876, B. cereus, ATCC 10987, B. megaterium, ATCC 9885, B. subtilis, NTSU 7, B. mycoides, MC, B. anthracis, MC, Clostridium sporogenes, MC, Escherichia coli, ATCC 10586, and Streptococcus faecalis, ATCC 10541.

The ATCC 10876 and 10987 strains of B. cereus were chosen as antigens as they were found to be ninety per cent effective in deleting Actinomycete-caused taste and odor in water (14).

The antisera were produced in rabbits of the two to three pound weight range, locally obtained, and without regard to sex. Essentially following procedures by Campbell, Garney, Cremer, and Sussdorf (7), twenty-six gauge disposable needles were used to inject dilutions of the antigen, equivalent to the number three McFarland nephelometer tube, into a marginal ear vein. The animals were injected a total of five times over an eighteen day

period, and were exsanguinated and sacrificed on the twenty-third day following the first injection.

Antigen preparation is also described by Campbell, et al. (7). The strains of B. cereus were cultured in tryptic soy broth (Difco Laboratories, Detroit, Michigan) for eighteen hours at 30 C, harvested by centrifugation at 10,000 rpm for ten minutes, then suspended and washed three times in 0.85 per cent sterile saline. The cells were then resuspended in 0.85 per cent saline, heated in a boiling water bath for thirty minutes after which time phenol crystals were added to give a 0.5 per cent solution and once again heated for thirty minutes. The finished antigens were tested for sterility by inoculation on tryptic soy agar plates and in thioglycolate broth (Difco Laboratories, Detroit, Michigan) on three consecutive days.

Blood for antiserum was taken via heart puncture from rabbits that were ether-anesthetized. The bleeding was accomplished with an eighteen gauge needle on either end of a four inch length of plastic tubing. One needle was used for the heart puncture; the other was inserted into the rubber stopper of an air evacuated 15 ml glass test tube. All materials except the test tubes were siliconized, and all equipment was sterile. Approximately 80 to 100 ml of whole blood can be obtained in this manner. The blood obtained was allowed to clot under refrigeration at 5 C

and the serum separated by centrifuging for ten minutes at 10,000 rpm.

Proteins were determined using the biuret reagent and procedure (7). A standard curve was prepared with 5x crystallized egg albumin and after a thirty minute reaction period, readings were made on the Spectronic 20, set at 540 mu.

Serum fractionation and gamma globulin separation was accomplished by saturated ammonium sulfate salting. Sulfates were subsequently removed by dialysis against 0.85 per cent sterile saline until tests with a two per cent barium chloride solution were negative (9).

The gamma globulin fractions of the antisera were conjugated at 35 C, for thirty minutes, according to Spendlove (21), by using fluorescein isothiocyanate (FITC) obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. A twenty mg/ml concentration of the powdered FITC was added for each gram protein in the total volume of antiglobulin. Final purification of the conjugated antiserum was by passage through a Pharmacia 18 inch Sephadex DEAE anion exchange column, using G-50 fine resin equilibrated with phosphate buffered saline (pH 7.2) (21). The final conjugate was diluted to a ten mg/ml protein concentration as is recommended for most bacterial labeling (19). Pooled whole serum from two rabbits, which

exhibited no titer against B. cereus, was conjugated for control serum. Goat-anti-rabbit sera (Colorado Serum Company, Denver, Colorado) was FITC conjugated for indirect labeling tests.

Titers on all sera, as shown in Table 1, were by standard tube agglutination methods (7).

Specificity testing necessitated the use of absorption procedures (7). All absorptions were reacted overnight at 5 C, using a cell suspension equivilent to the number three McFarland nephelometer tube and an equal volume of antisera. These heterologous absorptions were used in an attempt to remove those antibodies which caused non-specific fluorescence.

Both the direct slide technique, essentially described by Moody, Goldman, and Thomason (17), and the indirect slide technique, Weller and Coons (22), were used in testing reactivity and specificity of the B. cereus antisera. Slides were made of the desired organism, allowed to air dry, then fixed for ten minutes in acetone. At this stage, the slides could be used at once or stored for later use.

Smears for the direct technique were overlayered with normal rabbit serum for five minutes, to accomplish pre-inhibition as proposed by Bergman, Forsgren, and Swahn, (1) to decrease non-specific labeling. They were then washed with phosphate-buffered saline (PBS; pH 7.0), and

finally overlayered with the specific-labeled and pooled antisera for thirty minutes to one hour. The smears were then washed for two minutes each in PBS, carbonate-bicarbonate buffer, and distilled water. Finally, the cells were covered by a glass slip using a carbonate-bicarbonate buffered glycerol mounting fluid, as recommended by Pital and Janowitz (19). These prepared slides were observed for fluorescence with a Nikon SKE microscope (Nikon Inc., Garden City, New Jersey) equipped with a darkfield condenser (N.A. 1.50). A 200-w mercury arc lamp (Bausch and Lomb, Inc., Rochester, New York) was the light source. Green filters were used for visible lights, and Corning 5-58 filters were used for ultraviolet illumination. Nikon Y-8 yellow barrier filters were used for light filtration.

The indirect method essentially follows the procedure described for the direct technique. The primary difference is reaction of B. cereus cells or colonies with B. cereus antisera that was not FITC conjugated. This antigen-antibody complex was then reacted with a separate antiglobulin system, which was FITC conjugated. The separate system, goat-anit-rabbit sera, labeled the cells for fluorescence.

The membrane filter-fluorescent-antibody technique (MFFA) (11, 13) was used as the method to provide enumeration and identification of the B. cereus strains. The

results of the studies are given in Tables 4 and 5. This technique incorporated the use of black gridded filters, HABG 047, (Millipore Corporation, Bedford, Massachusetts) in glass filter bases and 250 ml glass funnels. Sterilized distilled water as well as sterilized, but otherwise untreated, pond water was used to dilute known concentrations of the organisms utilized in this study. These dilutions were filtered through the membrane filters; the filters were then removed and placed on tryptic soy agar plates. Incubation was carried out at 35 C for twelve hours. At the end of the incubation period the membrance filters were placed back on the filter base, a negative pressure applied, and the colonies were wetted with phosphate buffered saline (pH 7.0). The pressure was then allowed to equalize and the colonies were overlayered with normal rabbit serum for five minutes. This serum was removed by negative pressure and the colonies were again overlayered, this time, with the specific labeled antiglobulin for 15 to 20 minutes. The antiglobulin was removed by negative pressure, and the colonies washed free of excess antiglobulin with carbonate-bicarbonate buffer (pH 9.4). Glycerol mounting fluid, buffered (pH 9.0), was used to cover the colonies to prevent drying. Counts were made on a Nikon dissecting microscope (10 X magnification) using indirect illumination. Visible light counts gave the total colony

count and ultraviolet was used for the fluorescent number.

All bacterial colony counts were controlled by delivering 0.1 ml, of a viable cell suspension, of the appropriate dilution, on the center of a tryptic soy agar-filled petri plate. The aliquot was then spread evenly over the agar surface, by use of a sterile, glass, hockey stick-shaped spatula. Controls of vegetative cell and colony fluorescence were made by the addition of FITC-conjugated normal rabbit sera.

RESULTS AND DISCUSSION

Antisera produced by the ATCC 10876 and ATCC 10987 strains of Bacillus cereus, gave only moderate titers, as shown in Table 1. A titer as low as 1:128, shown in rabbit number five, was found to produce fluorescent reactivity. The antisera titers, even after conjugation, as evidenced by the fluorescent reactivity, Table 2, gave sufficiently good labeling results.

TABLE 1: RABBIT ANTI-BACILLUS SERA TITERS

Rabbit #	Antigen B. cereus #	Preinjection	18th day	23rd day
1	10876	1:8	1:256	1:512
2	10876	1:2	1:256	1:512
3	10876	0	1:256	1:1024
4	10876	0	1:64	1:128
5	10987	0	1:256	1:2048
6	10987	1:4	1:512	1:2048
*7	10987	1:32	1:2048	--
**8	None	1:2	--	--
**9	None	0	--	--

*Injection completed but antiserum not used due to elevated preinjection titer.

**Animals bled for "normal" control serum.

TABLE 2. F. A. REACTIVITY WITH B. CEREUS ANTISERA-SLIDE TECHNIQUE

Organism	F. A. Reaction-Direct* B. cereus Antisera ATCC # 10876	- 10987	F. A. Reaction Indirect** B. cereus Antisera ATCC # 10876	- 10987	Control
B. cereus ATCC 10876	2+	3+	3+	3+	0
B. cereus ATCC 10987	3+	3+	3+	4+	0
E. coli	0	0	0	(±)	0
Strep. faecalis	0	0	0	(±)	0
B. subtilis	3+	3+	4+	3+	1+
B. megaterium	3+	3+	3+	3+	(±)
B. mycoides	2+	2+	4+	3+	0
Clos. sporogenes	0	0	(±)	0	0.
B. anthracis	0	(±)	(±)	(±)	0

*Direct reactions are averages of five duplicate slides.

**Indirect reactions are averages of three duplicate slides.

Table 2 provides evidence that B. cereus vegetative cells reacted with the two antisera to give fluorescence. Lack of reactivity with Escherichia coli and Streptococcus faecalis, showed a trend toward family specificity. These particular organisms were chosen because of their use as water pollution indicators. Possibility of genus specificity

was provided by the negative reactions of Clostridium sporogenes. In these results only a slight increase in intensity, of the fluorescence, was noticed, with the indirect method. The direct labeling technique appears slightly more specific than the indirect. Specificity, as expected from the studies previously mentioned and as results with B. subtilis, B. megaterium, and B. mycoides showed, was a problem.

Table 3 data show the failure of heterologous absorption to resolve the lack of species specificity. Multiple absorption with the three species, that showed cross reactivity, was not successful. Absorption with the three organisms individually gave the same results. Homologous absorption with a mixed suspension of the two B. cereus, cells and antisera, rendered antisera that gave no fluorescence with the same B. cereus strains or with the B. mycoides strain. This same absorbed antisera showed positive results with B. subtilis and B. megaterium. It seems possible that the animals used had antibodies for the omnipresent B. subtilis, and perhaps for the B. megaterium, but pre-immunization titers were not studied.

Smears for the fluorescent slide method were made from eighteen hour old cultures, as older cultures showed considerable fragmentation and atypical cells. Time studies for the MFFA cultures gave best results at ten to twelve

TABLE 3. ABSORBED - B. CEREUS
ATCC # 10876 AND 10987 ANTISERA

1. *Absorbed with B. subtilis, B. mycoides, B. megaterium

Organism	Dilution	F. A. Reaction to Antisera 10876	10987	Controls**
B. cereus 10876	1:10	±	+	0
"	1:100	0	0	0
B. cereus 10987	1:10	+	+	0
"	1:100	±	+	0
"	$1:10^3$	0	0	0
B. subtilis	1:10	±	+	0
"	1:100	0	0	0
B. mycoides	1:10	+	+	0
"	1:100	±	0	0
"	$1:10^3$	0	0	0
B. megaterium	1:10	±	+	0
"	1:100	0	0	0

*All absorptions were reacted in a 1:1 cell to antisera ratio.

**Dilutions do not apply to controls.

0 = No Fluorescence
± = Trace (detectible)
+ = Good Fluorescence

TABLE 3. Continued

2. *Absorbed with B. subtilis

Organism	Dilution	F. A. reactions to Antisera 10876	10987	Control**
B. cereus 10876	$1:10^3$	±	±	0
"	$1:10^4$	0	0	0
B. cereus 10987	$1:10^3$	±	+	0
"	$1:10^4$	0	0	0
B. subtilis	$1:10^2$	+	+	0
"	$1:10^3$	±	±	0
"	$1:10^4$	0	0	0

3. *Absorbed with B. mycoides

Organism	Dilution	F. A. Reaction to Antisera 10876	10987	Control**
B. cereus 10876	$1:10^2$	±	±	0
"	$1:10^3$	0	±	0
"	$1:10^4$	0	±	0
B. cereus 10987	$1:10^2$	+	±	0
"	$1:10^3$	0	0	0
B. mycoides	$1:10^2$	+	±	0
"	$1:10^3$	±	±	0
"	$1:10^4$	0	0	0

TABLE 3. Continued

4. *Absorbed with B. megaterium

Organism	Dilution	F. A. Reaction to Antisera 10876	10987	Control**
B. cereus 10876	$1:10^2$	±	±	0
"	$1:10^3$	±	±	0
B. cereus 10987	$1:10^2$	±	±	0
"	$1:10^3$	0	0	0
B. megaterium	$1:10^2$	±	±	0
"	$1:10^3$	0	0	0

hours incubation. Incubation for longer periods yielded the typical colony spreading tendency of the genus Bacillus.

Tables 4 and 5 give the MFFA results for the B. cereus strains under investigation. These results indicate the efficiency of both enumeration and genus identification for B. cereus. Members of the genus Bacillus tested presented some difficulty, with the method, as they form dry, crinkled colonies that tend to become dislodged from the membrane, and float on the surface of both preinhibition and labeling sera. This difficulty was almost entirely

alleviated by wetting the colonies with PBS prior to the addition of the more viscid antisera. Wetting of the colonies also allowed the addition of antisera without the use of negative pressure, thus requiring a lesser volume for the contact time. Necessity of immediate colony counting due to rapid fading of fluorescence was, as reported (13), a problem. Allowing a thin film of the buffered glycerol to remain over the entire surface of the membrane was found to preserve the fluorescent intensity for at least ten minutes.

TABLE 4. MFFA - WITH B. CEREUS ATTCC # 10876 ANTISERA

Organism (s)	Calculated # Cells Added	Pre-Labeled Count*	Fluorescent Count*	Fluorescent Reaction	Plate Count*	F. A. Control
B. cereus 10876	30	48	41	2+	66	0
B. cereus 10987	30	39	[illegible]	2+	87	0
B. cereus 10876 + E. coli	30+15	68	19	1+	125	0
B. cereus 10876 +Strep. faecalis	30+15	120	14	1+	206	(±)
B. cereus 10876 + B. subtilis	30+15	22	29	3+	51	(±)

*Colony counts are averages from five duplicate counts.

TABLE 5. MFFA - WITH B. CEREUS
ATCC # 10987 ANTISERA

Organism (s)	Calculated # Cells Added	Pre-Labeled Count*	Fluorescent Count*	F. A. Reaction	Plate Count*	F. A. Control
B. cereus 10987	30	54	40	2+	89	(±)
B. cereus 10876	30	21	29	2+	63	0
B. cereus 10987 + E. coli	30+15	92	6	1+	160	(±)
B. cereus 10987 + Strep. faecalis	30+15	78	10	3+	71	1+
B. cereus 10987 + B. megaterium	30+15	60	55	3+	94	(±)

*Colony counts are averages from three duplicate counts.

B. cereus ATCC 10987 was added to jars of raw water obtained from a local surface pond. Each of five jars containing 50 ml of the water received dilutions of B. cereus amounting to 300 cells per ml of raw water. Only one duplicated count was made. This study was conducted to ascertain if the B. cereus could be demonstrated by the MFFA method from an untreated water. Results, Table 6, indicate that the normal flora of a raw water does not interfere with the fluorescent antibody labeling of the

B. cereus. After seventy-two hours, in a static condition, the total per cent of B. cereus increases, while the normal flora decreases, but insufficient tests were performed for valid assumptions.

TABLE 6. MFFA - BACILLUS CEREUS 10987
ADDED TO RAW POND WATER

Incubation Time (Hours)*	Dilution Raw HOH*	Pre-Label Count	Fluorescent Count	F. A. Reaction	Plate Count	F. A. Control
0***	10^6	24	6	1+	35	(±)
1	10^6	66	39	2+	49	(±)
18	10^6	26	8	2+	30	0
24	10^6	44	16	1+	86	(±)
48	10^3	17	14	2+	36	(±)
72	10^3	57	52	1+	75	(±)

*Zero incubation time has no added B. cereus.

**0.1 ml of each dilution was plated.

CONCLUSION

A review of this work indicates that the strains of Bacillus cereus employed as antigens provided antisera of sufficient titer for the fluorescent procedures used. The members of the genus Bacillus are readily labeled and both vegetative cells and colonies give good fluorescence.

Neither the absorption procedure nor the change in conjugation concentration provided a solution for the lack of antisera species specificity and more work will be necessary in this area.

Results indicate that the MFFA method is quite efficient for the rapid enumeration and provide a degree of generic identification for B. cereus in water.

It is plausible that the MFFA methods can be used in following the activity of B. cereus that has been added to water for treatment of taste and odor. Since the majority of Bacillus species are soil inhabitant, the use of this MFFA procedure could be considered to detect those organisms which may be added in the control of tastes and odors. Normal flora Bacillus species counts in waters would be expected to remain somewhat low and relatively stable.

Appendix C

Fecal Strep, an Index of Pollution as Determined by Fluorescent Antibody

The purpose of this study, utilizing the fluorescent antibody technique, is to evaluate the possibility of using the detection of the presence of S. faecalis as an indication of pollution. Also, various parameters are under consideration to determine if S. faecalis is a feasible organism to demonstrate fecal pollution. We are presently trying to make comparison studies of S. faecalis versus E. coli.

This fluorescent antibody study is being carried out with the use of five strains of Streptococcus faecalis; ATCC 349, ATCC 8043, ATCC 10541, MCS*, and a strain which was isolated from the sewage effluent at the local sewage plant. We will refer to the latter strain as N.I. (new isolate).

Production of antigens and antisera. Monospecific antibodies for the cell walls were produced in rabbits, essentially according to the methods described by Campbell, et. al. (1962) Thus, the O antigens for S. faecalis ATCC 349, ATCC 8043, ATCC 10541, and MCS* consisted of boiled and phenol (0.5%) treated suspensions of the listed strains.

The agglutinin titers of all the sera were determined by tube agglutination tests using the heat-killed antigens specific

for the antiserum. Also, live 18 hr. broth cultures were used as antigens and no significant difference was detected in the titers obtained. The resulting titers were as follows in Table 1: (These antigens were diluted to a concentration corresponding to a #3 McFarland nephelometer tube, 900 x 10^6 cells/ml.)

Table 1

Organism	Pre immune	Post immune	Tagged globulin
S. faecalis - ATCC 349 (N.T. 145)	0	512	256
S. faecalis - ATCC 8043 (N.T. 145)	4	128	64
*S. faecalis - ATCC 10541 (N.T. 148)	8	512	64
S. faecalis - MCS** (N.T. 147)	4	1024	32

* gave best fluroescent results

A two-fold dilution scheme was used. Readings were made after 2 hours at 45° C and after overnight refrigeration and centrifugation at approximately 5,000 rpm for 10 minutes. Saline controls were run under the same conditions, each giving negative results. No appreciable difference in the readings taken at 2 hrs. and those taken after overnight refrigeration were noticed. As can be seen from the table, some specific activity was lost from the time the specific antiserum was fractionated and labeled. The error is possibly in that there was a drop in pH of the conjugating

antisera after tagging was completed. According to Spendlove, the pH must be maintained at about 9.0 during the labelling process in order to insure maximum labelling of the globulin.

Presently, we are in the process of preparing antigens under more controlled conditions and will begin inoculation of rabbits with the five strains as soon as these antigens are produced. We are now utilizing a technique whereby the C polysaccharide is extracted by hot HCl (Lancefield) and we hope to obtain antisera with higher titres than those previously obtained.

Preparation of the globulin. The globulin fraction of each specific antiserum was obtained by fractionation by the ammonium sulfate method, essentially that of Campbell, et. al. (1966) Titres were obtained on these sera prior to conjugation with fluorscein isothiocyanate so as to compare the resulting titre of each with that obtained after conjugation.

Conjugation of globulins with fluorescein isothiocyanate. Each globulin fraction was labelled with FITC according to the method described by Spendlove (1966). Protein determinations were made on the antisera by the Biuret method as described by Gornall, et. al. (1949).

Fluorescent-antibody slide test. The direct method was used. Slides were prepared by streaking 18 hr. broth cultures of the

various strains of S. faecalis on to the slide and fixing them in acetone for approximately 15 minutes. Prior to streaking, these cells were centrifuged, the supernate decanted, and the cells were washed with phosphate buffered saline (PBS; pH 7.4 - 7.6). After fixation, the smears were overlayered with appropriately conjugated antisera (adjusted to be about 1 mg/ml protein). Negative controls were set up using conjugated normal rabbit serum. These slides were incubated for 1 hour in a 37° C incubator in a moisture chamber. Times less than 1 hour seemed to give less intense fluorescence. The slides were then washed in Coplin jars containing CO_3-HCO_3 buffer (pH 9.0). Glycerol mounting fluid (pH 9.0) was added, and then cover slips were placed on the slide. These slides were prepared with a twofold purpose in mind: (1) to become familar with the techniques involved, and (2) to determine the specificity and intensity of fluorescence of each antiserum. Table 2 shows results obtained.

Table 2

Test organism (S)	Conjugated Antiserum Used	Best obtained Fluorescent Intensity of Homologous Conjugate
S. faecalis - ATCC 349	145	1+
S. faecalis - ATCC 8043	146	2+
S. faecalis - MCS	147	2+
S. faecalis - ATCC 10541	148	2-3+
145 + 146	145	2+
145 + 147	145	2+
145 +148	145	1+
145 + N.I.	145	2+
146 + 145	146	2+
146 + 147	146	2+
146 + 148	146	2+
146 + N.I.	146	2+
146 + B. cereus - ATCC 10876	146	4+
146 + E. coli - ATCC	146	3+
147 + 145	147	2+
147 + 146	147	2+
147 + 148	147	2+
147 + N.I.	147	2+
148 + 145	148	2-3+

Table 2 (continued)

148 + 146	148	2-3+
148 + 147	148	2-3+
148 + W.I.	148	2-3+
148 + *E. coli* - ATCC	148	3+
148 + *B. cereus* - ATCC 10876	148	3+

Millipore Filter-Fluorescent Antibody Method. Water from a small pond was used as background. Plate counts were done on various dilutions of the pond water to ascertain a background population which was not too dense. A dilution 1×10^3 of the pond yielded an average of 40-50 colonies per ml. An overnight (18 hours) broth culture of S. faecalis was centrifuged at 8,000 rpm for 10 minutes, the supernate decanted, and the sediment washed with PBS (pH 7.2). Dilutions were carried out to 1×10^{10} cells. A 0.1 ml aliquot of each dilution was added, with 0.1 ml of 10^2 pond water, to a millipore filter funnel which contained a sterile 7HABG filter and approximately 50 mls of PBS. After application of negative pressure, the pad was transferred to TSA plates. This plate, as well as plates for other dilutions, were incubated at 37° C. Beginning at 12 hours, the filters were examined by fluorescence for the detection of Streptococcus faecalis. The quickest we have been able to identify the organism is 18 hours with a 2+ intensity. Also, we have encountered autofluorescence in several instances which needs to be eliminated. Other phases being looked at are an incubation temperature of 45.5° C and use of different media which may enhance growth. We have only used m-Enterococcus and TSA media. It cannot be concluded that TSA is better for rapid identification by

fluorescence, but our results seem to favor the use of TSA as the colonies appear about 2-4 hours sooner on TSA than on m-Enterococcus.

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